

Award Number: CDMRPL-16-0-BC151687

TITLE: **THE ROLE OF EXTRACELLULAR VESICLES IN METASTASIS**

PRINCIPAL INVESTIGATOR: **DR GABRIELA LOOTS**

CONTRACTING ORGANIZATION: **LAWRENCE LIVERMORE NATIONAL LABORATORY**
Livermore, CA 94550-9698

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2017		2. REPORT TYPE Annual		3. DATES COVERED 1 Oct 2016 - 30 Sep 2017	
4. TITLE AND SUBTITLE THE ROLE OF EXTRACELLULAR VESICLES IN METASTASIS				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER CDMRPL-16-0-BC151687	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Gabriela G. Loots E-Mail: lootsl@llnl.gov				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) LAWRENCE LIVERMORE NATIONAL LABORATORY Energy Livermore Office 7000 East Avenue Livermore, CA 94550-9698				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The proposed work addresses the dire need for new platforms for ESV isolation and precise, quantitative characterization of each cancerous ESV subpopulation's role in cargo transfer. Specifically, we aim to (1) optimize an existing microfluidic separation platform to efficiently isolate ESV subpopulations (from the cells and from each other) originating from breast cancer cell lines with a range of metastatic character, (2) engineer breast cancer cell lines with fluorescent and radiolabeled ESV sub-populations for individual tracking, and (3) use accelerator mass spectrometry (AMS), which allows extremely sensitive rare-molecule detection, to quantify low levels of tumor-derived RNA transferred via ESVs to osteoblasts. These bone cells represent the most common tissue target for breast cancer metastasis, and we will mimic ESV-mediated cancer invasion and metastasis by growing the cancerous and bone cells together in a trans-well cell co-culture system. The use of these molecular and physical tools in combination specifically to address cancer invasiveness and mechanisms of metastasis is unprecedented. This study will yield the first quantitative data on which ESV subpopulations (exosomes, MVs, or oncosomes) manipulate the tumor microenvironment, the ESV cargo transferred, and how this differs across the range of cell metastatic potential.					
15. SUBJECT TERMS None listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	3
2. Keywords.....	3
3. Accomplishments.....	3
4. Impact.....	12
5. Changes/Problems.....	13
6. Products.....	13
7.Participants & Other Collaborating Organizations.....	14

- **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The release of extracellular vesicles (ESVs) from high grade, aggressive forms of human cancer cells into their surroundings has become increasingly recognized as a feature of tumor biology, but what promotes ESV release, what cargo different ESV subpopulations carry, and what roles ESV contents have in tumor progression remains largely unknown. One hindrance to the lack of progress has been the scarcity of methods available to purify large quantities of ESV subpopulations intact, without cellular contaminants or without damaging the cargo. A second hindrance has been the lack of quantitative methods for measuring very small amounts of DNA and RNA transferred from tumor cells to the surrounding cells. As cancer progresses, the surrounding microenvironment co-evolves with the tumor through continuous paracrine cross-communication, thus creating a dynamic signaling circuitry that promotes cancer initiation, growth, drug resistance, metastasis and ultimately organ failure and death. The stromal components that include endothelial cells, pericytes, fibroblasts, various classes of leukocytes, and extracellular matrix are likely to receive ‘executive signals’ from the tumor in the form of proteins, mRNAs, ncRNAs, miRNAs and DNA to promote phenotyping changes in the stromal components that benefit the tumor. If we can detect the signals propagated from the tumor cells to the stroma, we can begin to formulate new testable hypotheses on how cancer cells manipulate their microenvironment to develop an aggressive phenotype. To address these shortcomings, the project has three specific aims:

- Aim 1.** Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].
- Aim 2.** Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.
- Aim 3.** Use accelerator mass spectrometry (AMS) technologies to quantify low levels of tumor-derived RNA transferred via ESVs to osteoblasts, and characterize their functions in promoting invasion.

- **KEYWORDS:**

Breast cancer, extracellular vesicles, exosome, MDA-MB-231, MCF7, MCF10A, metastasis

- **ACCOMPLISHMENTS:**

- What were the major goals of the project?

	Year 1	Year 2	Year 3
<i>Specific Aim 1. Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].</i>			
<i>Task 1A: Verify microfluidic separation performance of ESVs from host cells and debris using existing acousto-fluidic devices with each of the 3 breast cancer cell lines</i>			
	1a: Generate mixed cell-vesicle samples by growing cells in serum-starved media; process samples through separation device at a range of flow and pressure-field parameters and assess separation efficiency and purity by cell counting, SEM and fluorescence		

	microscopy and qPCR.		
Task 1B: Determine optimal separation strategy for oncosome population in each of the 3 breast cancer cell lines; verify bead-complexed separation of populations			
	1b: In tandem with Task 1a, for each cell line, determine whether oncosomes are best grouped with “large” (cells) or “small” group (microvesicles and exosomes), i.e. conditions at which largest fraction of oncosomes are recovered.	Label subpopulation-specific ESV surface markers (e.g. CD63) with antibody-derivatized polystyrene microspheres and verify purity and separation efficiency of resulting subpopulation separation by qPCR and SEM.	
<i>Milestone #1: Tabulate separation parameters (flow rate, actuation voltage and frequency) for purifying ESVs vs. host cells/debris for each of 3 cell lines; publication on acoustic device performance</i>			
Task 1C: Design and fabricate acoustic separator chips specifically optimized for isolation of ESV subpopulations			
		1c: From results of Tasks 1a and 1b, generate new photo-masks, and fabricate new microfluidic devices, optimally configured to isolate ESV subpopulations, ideally in a single pass through the device.	
<i>Milestone #2: Fabricate 30-50 new microfluidic devices based on optimized design parameters.</i>			
Task 1D: Verify isolation and purification of multiple ESV subpopulations by optimized acoustofluidic device			
		1d: Using mixed cell-vesicle samples grown from serum-starved media, pass samples through optimized devices and assess separation efficiency, purity and recovery.	Continue using acoustic devices to generate pure ESV samples for supporting the efforts in Tasks 2 and 3.
<i>Milestone #3: A functional automated separation platform capable of rapid recovery of individual ESV subpopulations from bulk cell culture samples.</i>			
<i>Milestone #4: Publication reporting results of platform development and novel findings on ESV shedding rates and quantities in breast cancer cell lines of different metastatic character.</i>			
Specific Aim 2: Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.			
Task 2A: Engineer [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)] cell lines to express fluorescent markers that discriminate exosomes from			

microvesicles.			
2a: Create DNA constructs that express CD63 or CD9 [exosome markers] fusion proteins with mKate, a far red fluorescent protein and CD40 or CD63 [microvesicle marker] fusion proteins with eGFP, an enhanced green fluorescent protein	Where available fusion protein clones will be purchased, transfected into MDA-MB-231; MCF7; MCF10A cells and selected for stable transfected cell lines. If not available for purchase, the full length cDNA clones will be obtained from IMAGE and subcloned to insert mKate or eGFP in frame to create fusion constructs	Stable transfected cell lines with one exosome and one microvesicle specific markers will be characterized using imaging, western blots, and immunoprecipitation to confirm the location of the fluorescent protein.	
<i>Milestone #5: Create new MDA-MB-231, MCF7, MCF10A subclonal cell lines that express fluorescent markers that allow us to discriminate between exosomes (red) and microvesicles (green). Each breast cancer cell line will be positive for 2 transgenes [mKate+; eGFP+]</i>			
Task 2B: Engineer [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)] cell lines to express a transgene for uracil phosphoribosyltransferase [UPRT].			
2b: Transfect DNA construct that expresses UPRT into MDA-MB-231; MCF7; MCF10A cells and select for stable cell lines.		MDA-MB-231; MCF7; MCF10A cells lines expressing mKate or eGFP fusion proteins will be transfected with UPRT vectors, and select for triple transgenic lines [mKate+; eGFP+; UPRT+]	
<i>Milestone #6: Create new MDA-MB-231, MCF7, MCF10A subclonal cell lines that express fluorescent markers that allow us to discriminate between exosomes (red) and microvesicles (green) and also express UPRT. Each breast cancer cell line will be positive for 3 transgenes [mKate+; eGFP+; UPRT+]</i>			
Specific Aim 3: Use Accelerator Mass Spectrometry (AMS) technologies to quantify low levels of tumor derived RNA transferred via ESVs to osteoblasts, and characterize their functions in promoting invasion			
Task 3A: Determine if cancer cells derived microvesicles or exosomes carry RNA			
		3a. Different ESV subpopulations derived from triple transgenic cancer cell lines and cultured with ¹⁴ C-thiouracil will be isolated using microfluidic device and the ¹⁴ C-level will be quantified using AMS. Since only RNA will be labeled with ¹⁴ C, only	Quantify the amount of RNA packaged in ESV in the 3 triple transgenic cancer cell lines cultured with ¹⁴ C-thiouracil. Optimize culture conditions to enrich for ESV populations that carry RNA, to obtain sufficient RNA for

		ESV populations positive for ^{14}C will be used to isolate RNA and sequence the RNA	sequencing
<i>Milestone #7: Determine which ESV subpopulation has mRNA cargo</i>			
Task 3B: Determine if cancer cells derived microvesicles or exosomes are taken up by osteoblasts			
3b:			3b: Triple transgenic lines created in 2b will be co-cultured with osteoblasts, at different time points [6 hours, 24 hours, 48 hours, 96 hours] the RNA will be isolated from osteoblasts and ^{14}C will be quantified to determine if RNA was transferred from cancer cells to the bone cells. RNA will be further isolated and sequenced
<i>Milestone #8: Identify whether tumor cells package mRNA randomly into ESVs, or whether there is a rationale and metastatic tumors package 'unique' mRNA species that are more likely to influence their environment</i>			
<i>Milestone #9: Publication reporting results of ESV differences in transferring RNA to osteoblasts, among breast cancer cell lines with different metastatic character.</i>			

- What was accomplished under these goals?

Specific Aim 1. Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].

Task 1A: Verify microfluidic separation performance of ESVs from host cells and debris using existing acousto-fluidic devices with each of the 3 breast cancer cell lines

1a: Generate mixed cell-vesicle samples by growing cells in serum-starved media; process samples through separation device at a range of flow and pressure-field parameters and assess separation efficiency and purity by cell counting, SEM and fluorescence microscopy and qPCR.

Towards our goal of developing microfluidic methods of ESV we have:

- ✓ Thoroughly characterized the separation efficiency of three different cell types.
- ✓ Assessed different methods for exosome and micro vesicle quantification.
- ✓ Demonstrated moderate separation using surrogate particles to determine feasibility of separation exosomes bound to immunocapture beads and microvesicles.

First, we designed and manufactured a new batch of microfluidic acoustophoretic separators. This design iteration tested several strategies to maximize particle residence time within the chip for improving separation while maintaining high volumetric flow. Next, we fully characterized these devices by determining the resonant frequency, and flow focusing characteristics with 7 μm

polystyrene beads. This maintenance and development is essential to ensure we have an adequate stock of well calibrated devices for biological tests.

We further quantified the separation of three different cell types into cell outlet to determine the optimal conditions to remove cells and cell debris from vesicle samples. Overall, we found slight differences in the different cell types, with MCF7As requiring the highest voltage to achieve separation.

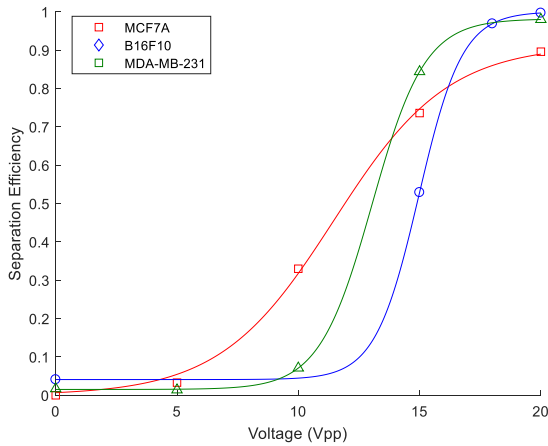


Figure 1. Separation efficiency of cells and cell debris from smaller constituents (extracellular vesicles). Separation efficiency is defined as the number of cells exiting from the Large Particle Outlet (LPO) scaled by the total number of cells recovered after separation. The experimental data is fit to a sigmoid curve. Depending on the cell type different voltages are required to remove cells and cell debris. Samples are run at 100 μ l/minute.

To evaluate the performance of our acoustic separation on extracellular vesicles we first needed to develop methods to measure different populations of vesicles. Therefore, a significant effort was invested in identifying ways to quantify and measure vesicles.

To identify exosomes we first concentrated exosomes using standard techniques of 0.2 μ m filtration and ultracentrifugation. Then, we used a lipophilic dye to fluorescently label all vesicles. Next, immunological CD9 and CD63 beads were incubated with the exosomes, and samples were quantified by FACS.

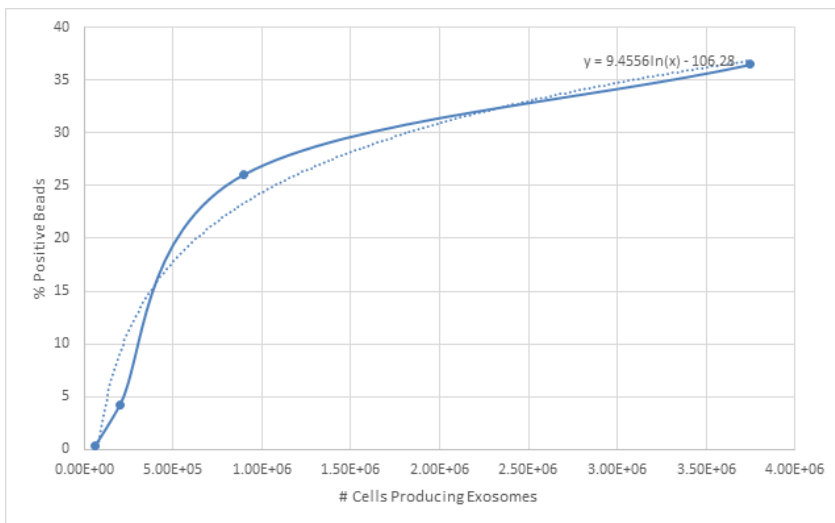


Figure 2: Calibration curve of positive signal versus number of cells using bead method to quantify exosomes.

Using these methods, we ran exosome samples under conditions to remove cell and cell debris (100 μ l/minute flow rate, 20Vpp) and show that exosomes exit out of the vesicle Small Particle Outlet (SPO). Preliminary results indicate that exosomes and cells can readily be separated using this technique.

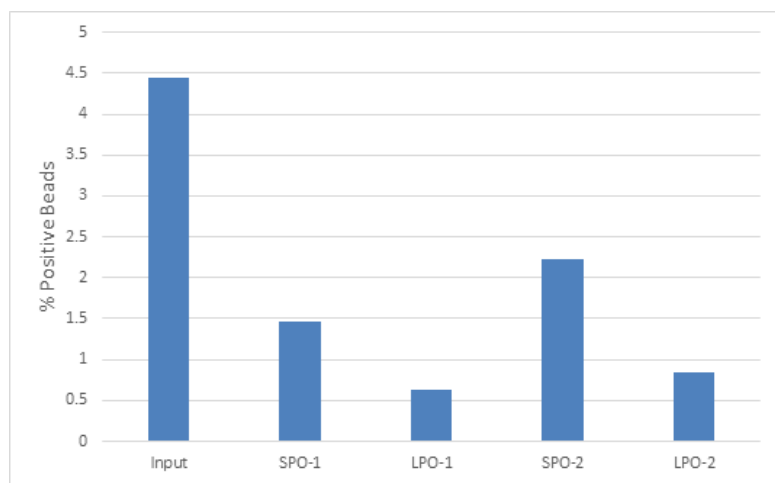


Figure 3: Relative output from two different runs through the acoustophoretic separator at conditions for cell separation. The SPO fractions have more positive beads than the LPO fraction, indicating that exosomes will exit from the SPO while cells will exit from the LPO.

Task 1B: Determine optimal separation strategy for oncosome population in each of the 3 breast cancer cell lines; verify bead-complexed separation of populations

1b: In tandem with Task 1a, for each cell line, determine whether oncosomes are best grouped with “large” -(cells) or “small” group (microvesicles and exosomes), i.e. conditions at which largest fraction of oncosomes are recovered.

In Task 1B we proposed to investigate separation of oncosomes, however in all the analyses described above, we were not able to ‘find’ oncosomes. In the literature these types of vesicles are very loosely defined, therefore, we are now investigating methods to further quantify the sizes of the different vesicle populations, and determine whether we can distinguish uniquely sized groups. We have initially focused on using Dynamic Light Scattering (DLS), as statistical method which measures light scatter from particles to determine particle sizes present in a sample. We used differential centrifugation to isolate microvesicles and exosomes from conditioned cell media by ultracentrifugation at 10,000G for 90 minutes. The pellet was then resuspended to 100X concentrated and considered the microvesicle fraction. The supernatant was then filtered through a 0.2µm filter and spun down at 200,000G for 2 hours as the exosome fraction. MCF7 cells show the presence of 200nm and 1000nm particles, while 4T1 cells have a broader peak from 200-400nm. These results are in line with reported values for microvesicle sizes. The exosome fraction was too dilute to measure using this technique, however, preliminary results with exosome fractions isolated from B16F10 cells show a moderate signal. Therefore, we hypothesize the rate of exosome production may vary dramatically between different cell types. Our results thus far indicate that DLS will be a valuable tool to quantify the size of isolated vesicle populations, specifically for microvesicles, and potentially for exosomes as well. At this point however we have no evidence of larger particles that may qualify as oncosomes. We will continue to explore oncosome isolation in FY18.

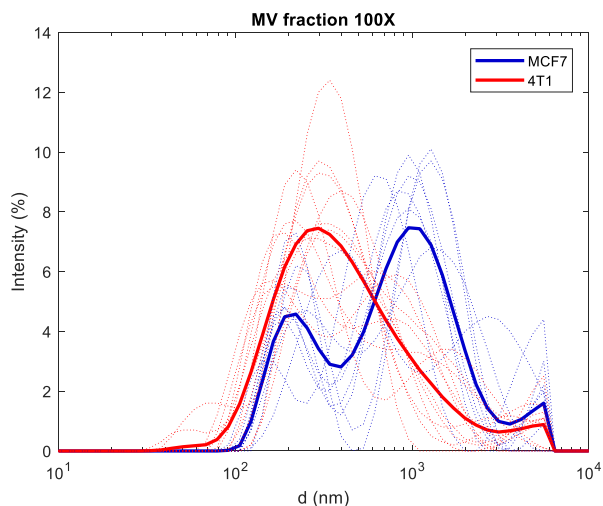


Figure 4: Dynamic light scattering results from microvesicles collected from MCF7 cell sand 4T1 cells. Thick lines indicate mean values taken over multiple runs (dotted lines).

In task 1B we proposed isolating exosomes via immunocapture beads from other vesicle populations. In the proposed work we intended to use large 10 μm immunocapture beads, however, beads that are readily available with specific exosome markers are only 2.7 μm . To determine if it is feasible to use these smaller beads to isolate exosome bead complexes we used 2 μm fluorescent beads to mimic the 2.7 μm exosome-bead complexes, and 1 μm beads to mimic microvesicles. We further decided to use this model system due to the challenges with measuring different vesicle populations as discussed above.

We expect this model system to underestimate the ability of our system to separate real samples. This is due to two reasons: Firstly, since the acoustic radiation is a volumetric force, a 2.7 μm particle will experience 2.5 times greater force than a 2.0 μm particle. Secondly, we are using 1 μm polystyrene beads to mimic 200nm-1 μm microvesicles which have very different acoustic properties. The acoustic force depends on the particle compressibility and density. Since vesicles are more compressible than beads, the acoustic force on a vesicle will be approximately 60% of the force on a polystyrene bead of the same size. Taken together, this means that the bead-complexed exosomes will move more strongly towards the node position, and the un-complexed microvesicles will move less than their surrogate bead particles used in this test. We expect this to result in better results for real samples than predicted using bead models.

After testing different voltages and flow rates, we find that at 25 $\mu\text{l}/\text{minute}$ and 20V we can achieve moderate separation of 1 and 2 μm beads. Due to the reasons discussed, we believe that this demonstration of moderate separation of beads will result in good separation of bead-complexed exosomes and microvesicles. Furthermore, we show that we can use the same device to perform this separation as devices used for removing cells and cell debris, simply by changing operating conditions.

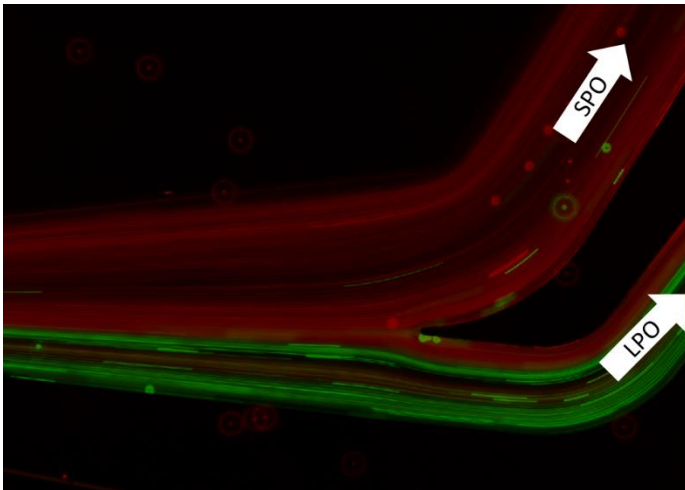


Figure 5: Testing with model system of 1 (red) and 2 (green) μm fluorescent beads to mimic unbound microvesicles, and immunological bead bound exosomes. At 20Vpp and 25 $\mu\text{l}/\text{min}$, large 2 μm green beads exit primarily out of the LPO while small 1 μm beads exit primarily out of the SPO.

Beyond what was initially proposed, we have also determined that the most beneficial result and most useful tool would be to generate a method to isolate exosomes and microvesicles without requiring labeling to obtain functional vesicles post separation. We further hypothesized that microfluidic techniques can be less destructive than standard ultracentrifugation practices to isolate different sized vesicles. Therefore, in conjunction with the above efforts we have also begun preliminary testing to determine if it will be possible to separate vesicles without immunological beads.

Sample	Flow rate (μlpm)	Voltage (Vpp)
Cell and cell debris and vesicles (all sizes)	100	15
Exosomes coupled to beads (2.7 μm) and microvesicles ~1 μm	25	20
Unbound exosomes and microvesicles	13 ulpm	25V *experiments in progress

Table 1. Summary of optimized parameters for separation of different samples.

Efficiency of extracellular vesicle isolation using acoustic separation vs. standard ultracentrifugation techniques. In order to compare acoustic separation of exosomes and microvesicles to standard ultracentrifugation-based isolation techniques, we quantified total protein content from large and small extracellular vesicles collected using both separation methods. Vesicles were derived from approximately 1.1×10^7 MCF-7 breast cancer cells post 48hr serum starvation. Exosomes from the small particle outlet (SPO) and microvesicles from the large particle outlet (LPO) were collected via acoustic separation from conditioned media at a flow rate of 13 μl/min at 24 V. In parallel, exosomes were collected by ultracentrifuging conditioned media at 200,000 x g for 2 hours at 4 degrees C post filtration through a 0.2 μm filter. Additionally, microvesicle fractions were collected by ultracentrifugation for 10,000 x g for 90 minutes at 4 degrees C. All fractions were resuspended in PBS + 1% DMSO and stored at -80 degrees C for further analysis.

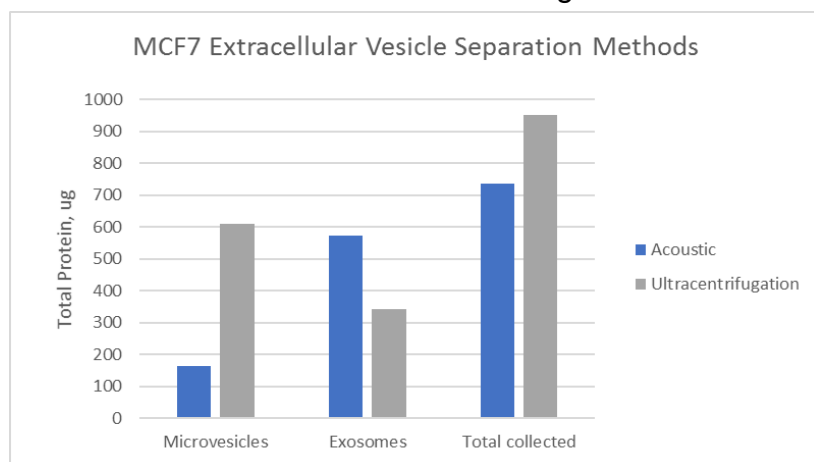


Figure 6. Comparison of extracellular vesicle separation techniques via total protein quantification. Acoustic separation microvesicles and exosomes fractions correspond to outputs collected from the LPO and SPO, respectively.

Both techniques yielded quantifiable amounts of extracellular vesicle-derived protein, however there was a loss of approximately 25% when using acoustic separation (Fig. 6). This is likely attributed to sample loss from processing through the acoustic separation apparatus, combined with losses incurred during post-processing ultracentrifugation to concentrate samples. Additionally, there is likely contamination of microvesicles in the SPO-collected (exosome) fraction. While the total protein yield of exosomes collected is higher from acoustic separation compared to standard ultracentrifugation, future western blotting using microvesicle and exosome specific markers will help to evaluate purity of fractions collected (Table 2).

Microvesicle	Exosome
CD147	CD63
TF	CD9

Table 2. Microvesicle and exosome-specific proteins for MCF7-derived vesicles¹⁻⁴.

Specific Aim 2: Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.

Task 2A: Engineer [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)] cell lines to express fluorescent markers that discriminate exosomes from microvesicles.

2a: Create DNA constructs that express CD63 or CD9 [exosome markers] fusion proteins with mKate, a far red fluorescent protein and CD40 or CD63 [microvesicle marker] fusion proteins with eGFP, an enhanced green fluorescent protein

Towards our goal of engineering cell lines we have:

- ✓ Engineered MDA-MB-231 cell line to express CD9-, CD63-, and CD81-GFP fusion proteins.
- ✓ Shown that engineered cells can be sorted by FACs
- ✓ Shown fusion protein expression by Western blot

Upon design of the fusion protein constructs, it was found that the inconsistent expression of specific markers on exosomes may pose future limitations to both the ability to capture and localize exosome transport. Classic exosome markers such as Tsg101, Rab-5b, and CD-63 were shown to produce variable expression depending on origin of the exosome [Yoshioka et al.2013]. To circumvent this heterogeneity, we have created a plasmid construct containing GFP fusion proteins for 3 common exosome tetraspanin markers (CD9, CD63, and CD81) [Andreu & Yanez-Mo 2014] in tandem (pLLNL-exo-GFP) in order to both increase fluorescent intensity per exosome and label a more comprehensive population of exosomes (Fig 7a). A MDA-MB-231 cell line stably expressing pLLNL-exo-GFP has been created and validated for GFP expression *via* flow cytometry (Fig 7b) as well as robust expression of a fusion CD-63 with increased molecular weight (Fig 7c). Further research will include probing fluorescence of transgenic EV populations as well as creation of comparable MCF7 and MCF10a cell lines as well.

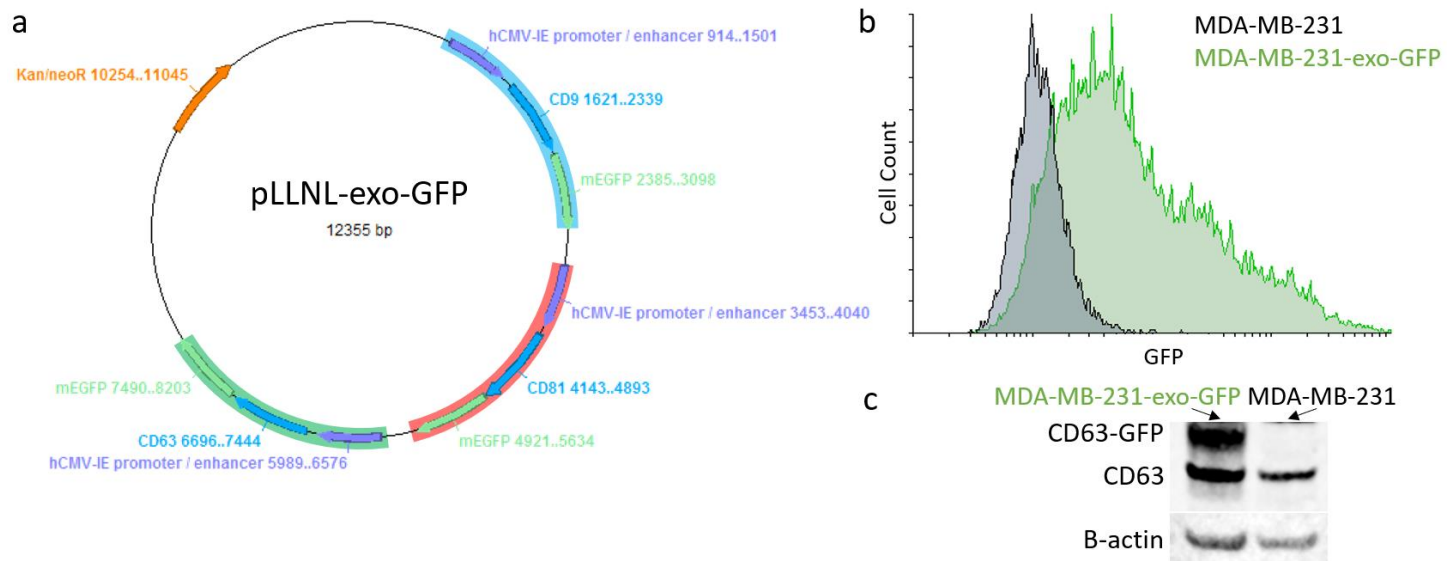


Figure 7: Genetic labeling of exosome populations. Transgenic construct with 3 tandem fusion proteins (a). Cell sorting of MDA-MB-231 transgenic stable line (b), protein expression of fusion protein, in transgenic MDA-MB-231 line MDA-MB-231-exo-GFP (c).

While endogenous labeling has been successful for exosome labeling, the microvesicle population lacks consistent protein markers to create for fusion marker creation. To circumvent this shortcoming, lipophilic membrane dyes (DiO, DiL, DiB) have proven effective means of labeling total EV

populations with fluorescence. Future efforts will be focused on staining total EV populations with a red dye and then denoting microvesicles as vesicles with solely red label.

Milestones:

Tabulate separation parameters (flow rate, actuation voltage and frequency) for purifying ESVs vs. host cells/debris for each of 3 cell lines

Create new MDA-MB-231, cell lines that express fluorescent markers that allow us to discriminate exosomes from cells and microvesicles in the microfluidic device.

- **What opportunities for training and professional development has the project provided?**

Kelly Martin is a graduate student from Georgetown University who is conducting her Ph.D work at LLNL under Dr. Loots' mentorship. She has allocated approximately 30% of her research efforts towards this project. She has been involved with the isolation, characterization and fluorescent labeling of exosomes and microvesicles derived from various breast cancer cell lines. She has also presented research and received insightful feedback from the research community in a variety of forums including the Annual Cancer Research Symposium held at UC Davis Comprehensive Cancer Center and the American Association for Cancer Research (AACR) annual meeting.

Daniel Sosebee is a pre-college student. He spent 3 months this summer [May 2017- July 2017] as an intern under Drs. Shusteff and Fong's mentorship. He thoroughly tested and characterized over 10 acoustophoretic separation chips that were subsequently used to isolate different extracellular vesicle populations. He determined the optimal frequency for separation, and quantified the effect of different voltages on separation efficiency. He further assisted in identifying the conditions necessary to separate cells and cell debris from extracellular vesicles.

- **How were the results disseminated to communities of interest?**

- *Nothing to report this period*

- **What do you plan to do during the next reporting period to accomplish the goals?**

- *During the next year we will finalize the generation of the transgenic lines, and initiate purification and characterization of ESV populations from the 3 breast cancer cell lines, we will also initiate the optimization experiments for C14 labeling of RNA, to determine the limits of detection.*

- **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

- **What was the impact on the development of the principal discipline(s) of the project?**

- *Nothing to Report*

- **What was the impact on other disciplines?**

- *Nothing to Report*

- **What was the impact on technology transfer?**

- *Nothing to Report*

- **What was the impact on society beyond science and technology?**

- *Nothing to Report*

- **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

- **Changes in approach and reasons for change**

- *None*

- **Actual or anticipated problems or delays and actions or plans to resolve them**

In Task 1B we proposed to investigate separation of oncosomes, however in all the analyses described above, we were not able to 'find' oncosomes. At this point we have no evidence of larger particles that may qualify as oncosomes. We will continue to explore oncosome isolation in FY18. If by the end of FY18 we still can't isolate oncosomes, the rest of FY19 will focus only on exosomes and microvesicles for functional characterization.

We hoped to have all 3 cell lines transgenic made by the end of the 1st year, however, we have some difficulties in optimizing signal when we co-transfected 3 plasmid with 3 markers, we had to switch our strategy to adding 3 fusion proteins in tandem on one plasmid to enhance the GFP signal and be able to detect it by FACS analysis. Now we can continue to make the remaining 2 cell lines transgenic, and proceed with the characterization as initially proposed.

- **Changes that had a significant impact on expenditures**

- *No*

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- *No*

- **Significant changes in use or care of human subjects**

- *Not applicable*

- **Significant changes in use or care of vertebrate animals.**

- *Not applicable*

- **Significant changes in use of biohazards and/or select agents**

- *Not applicable*

-

- **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- *Nothing to Report*

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

- *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	<i>Gabriela G Loots</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	0000-0001-9546-5561
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Loots was in charge of overseeing the project and collaboration with engineering group, met with team regularly [weekly] to discuss experimental design, data analysis, troubleshooting and future directions</i>
Funding Support:	<i>n/a</i>

Name:	<i>Maxim Shusteff</i>
Project Role:	<i>Co-PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Shusteff was in charge of overseeing the engineering component of this project, met with team regularly [weekly] to discuss experimental design, data analysis, troubleshooting and future directions</i>
Funding Support:	<i>n/a</i>

Name:	<i>Erika Fong</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>3.5</i>
Contribution to Project:	<i>Dr. Fong conducted all engineering, microfluidic experiments, met with the biologists regularly, optimized experimental design, collected data, analyzed data, troubleshooting</i>
Funding Support:	<i>n/a</i>

Name:	<i>Nicholas Hum</i>
Project Role:	<i>Biomedical scientist</i>
Nearest person month worked:	2.5
Contribution to Project:	<i>Mr. Hum in a biologist, he conducted cloning, culturing the cells, transfecting the cells, carrying our FACs analysis, isolating ESV via centrifugation</i>
Funding Support:	<i>n/a</i>

Name:	<i>Kelly Martin</i>
Project Role:	<i>Graduate Student</i>
Nearest person month worked:	3
Contribution to Project:	<i>Ms. Martin is a graduate student and has performed ESV isolation via ultracentrifugation, ESV characterization and cell culture.</i>
Funding Support:	<i>Livermore Graduate Scholar Fellowship</i>

Name:	<i>Daniel Sosebee</i>
Project Role:	<i>Summer Intern</i>
Nearest person month worked:	1
Contribution to Project:	<i>Daniel tested and optimized chips for microfluidics.</i>
Funding Support:	<i>n/a</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - *Nothing to Report"*
- **What other organizations were involved as partners?**
 - *Nothing to Report*